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Zebra Mussel Spawning Is Induced in Low Concentrations of Putative Serotonin Reuptake Inhibitors

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Abstract. Serotonin (5-hydroxytryptamine, 5-HT) and its receptor ligands induce both oocyte maturation and spawning in zebra mussels (*Dreissena polymorpha*). The selective serotonin reuptake inhibitors (SSRIs) fluvoxamine (''Luvox''), fluoxetine (''Prozac''), and paroxetine (''Paxil'') are commonly prescribed drugs for the treatment of depression in humans. They act to increase 5-HT neurotransmission by inhibiting reuptake transport proteins at synapses. I tested the efficacy of these drugs at inducing spawning in zebra mussels. All three compounds induced spawning in both sexes at concentrations lower than that for 5-HT itself. Fluvoxamine was particularly potent, inducing spawning in 100% of both sexes at 10^{-5} and 10^{-6} *M*. The concentration that induced a significant percentage of animals to spawn was as low as 10^{-9} *M* for males and and 10^{-7} *M* for females. The lowest concentration of fluvoxamine to induce spawning was 10^{-8} *M* for females (40%) and 10^{-10} *M* for males (20%). Gametes spawned in fluvoxamine (10^{-5} *M* and lower) were viable, and swimming trochophores were formed within 20 hours. Fluoxetine was also an effective spawning inducer, causing 100% of males to spawn at 5×10^{-6} *M*. The concentration of fluoxetine required to induce a significant percentage of spawning was as low as 5×10^{-8} *M* for males and 5×10^{-6} *M* for females. In both fluvoxamine and fluoxetine, more than 60% of the males spawned within the first hour of exposure. In contrast, paroxetine was a weak spawning inducer. At concentrations of 10^{-5} and 10^{-6} *M* it induced significant, but low (50% and 40%, respectively) percentages of males to spawn. Paroxetine did not induce significant spawning in females. Thus, fluvoxamine, fluoxetine, and paroxetine can induce spawning at low concentrations, and fluvoxamine is the most powerful spawning inducer in any bivalve. These may be useful agents for stimulating invertebrate serotonergic mechanisms without applying exogenous 5-HT, and they are potentially important in bivalve aquaculture. Moreover, these results suggest, for the first time, the presence of 5-HT reuptake transporters in bivalve molluscs.

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Introduction

Serotonergic mechanisms regulate a wide variety of physiological functions in molluscs. Amongst bivalve molluscs, reproductive processes including oocyte maturation (Hirai *et al.*, 1988; Krantic *et al.*, 1991; Fong *et al.*, 1994a; Gobet *et al.*, 1994), spawning (Hirai *et al.*, 1988; Ram *et al.*, 1993), and parturition (Fong and Warner, 1995; Fong *et al.*, 1996a) are regulated by serotonin (5-hydroxytryptamine, 5-HT) or a 5-HT-like compound. Exogenous application of 5-HT and 5-HT receptor ligands such as 8-OH-DPAT and alpha-methyl-5-HT induce spawning in a number of marine and freshwater bivalves (Gibbons and Castagna, 1984; Ram *et al.*, 1993; Fong *et al.*, 1993, 1996b). The serotonin pharmacology of spawning has been recently elucidated in the exotic zebra mussel, *Dreissena polymorpha* (Fong *et al.*, 1993, 1994b). Both male and female zebra mussels spawn when exposed to 10^{-4} *M* and 10^{-3} *M* 5-HT.

Fluvoxamine (5-methoxy-4′-(trifluoromethyl)valerophenone(E)-O-(2-amimoethyl)oxime maleate), fluoxetine (*N*-methyl-3[*p*-trifluoromethylphenoxy]-3-phenylpropylamine), and paroxetine {(-)-trans-4R-(4′-fluorophenyl)-3S-[93′,4′-methylenedioxyphenoxy)methyl] piperidine hydrochloride hemihydrate} are commonly prescribed antidepressants (''Luvox,'' ''Prozac,'' and ''Paxil,'' respec-

tively) in humans; they increase 5-HT neurotransmission by inhibiting 5-HT reuptake transporters (Fuller 1994; Garcia-Colunga *et al.*, 1997). Since these selective serotonin reuptake inhibitors (SSRIs) increase endogenous 5-HT neurotransmission in some species, I tested the effects of these drugs on spawning in zebra mussels. The results revealed that the three drugs, especially fluvoxamine and fluoxetine, are powerful inducers of spawning in zebra mussels, and suggest the presence of 5-HT reuptake transporters in bivalve molluscs.

Materials and Methods

Zebra mussels were collected in July 1996 and June 1997 from Conesus Lake (42° 45′ N, 78° 45′ W), Geneseo, Livingston County, New York. During the 1996 collection, the water temperature of the lake was 23°C. Mussels were transported to the laboratory and acclimated to 10°C in an incubator by reducing the temperature from 23°C at a rate of 2°C/day. Mussels were maintained without added food for 2 weeks until testing. These mussels were tested with fluoxetine only. In 1997, water temperature was 20°C during collection. Mussels were maintained without added food in a 10°C incubator and used within 3 days of collection. These animals were tested with fluvoxamine, fluoxetine, and paroxetine. Animals ranged from 13–32 mm in shell length. Fluoxetine, serotonin creatinine sulfate (both from Sigma Chemical Co., St. Louis, MO), and paroxetine (SmithKline Beecham, Philadelphia, PA) were dissolved in lake water. Fluvoxamine (Solvay-Duphar, Weesp, The Netherlands) was dissolved in 100% ETOH.

All experiments were carried out in 20-ml glass vials (1 mussel/vial) at room temperature (22°–25°C). Initially, all mussels were acclimated in either 4.5 or 9.0 ml of lake water for 20–30 min before addition of any drug. After the acclimation period, 0.5 or 1.0 ml of drug was added. Thus the final concentrations were 10-fold lower than the added concentrations. All experiments had a negative control (lake water alone or 0.1% ETOH) and a positive serotonin (10^{-3} *M*) control. Mussels were observed for evidence of spawning, and questionable spawnings were confirmed by microscopic analysis of water. In most cases, spawnings were easy to detect. Males released streams of sperm, which resulted in in cloudy water soon after. Females released oocytes in intermittent bursts; oocytes were easily seen on the bottom of vials. Experiments were run for 4 h, after which all non-spawners were dissected and their gonads examined microscopically to determine sex and reproductive maturity (Ram *et al.*, 1993). Since it is impossible to ascertain zebra mussel sex and maturity prior to experiments, the number of animals of each sex in various experimental groups varied from experiment to experiment, but each group initially consisted of at least 12 mussels. Results were analyzed statistically using Fisher's exact test (Sokal and Rohlf, 1981), and null hypotheses were rejected where $P < 0.05$.

Results

Figure 1A shows the dose-response curve for spawning in fluvoxamine. Males showed statistically significant percentages of spawning at 10^{-9} to 10^{-5} *M* (Fisher's exact test, $P < 0.0001$ for all concentrations compared with negative control). Females showed significant spawning in fluvoxamine concentrations from 10^{-7}–10^{-5} *M* (Fisher's exact test, $P < 0.003$–0.0001). As expected, 5-HT induced a high percentage of spawning compared with negative controls (Fig. 1B). At concentrations from 10^{-9} to 10^{-6} *M*, more than 60% of males spawned within the first hour, and of these, most spawned within 30 min (Fig. 2A). Females always took longer to spawn, never achieving maximum spawning in the first hour (Fig. 2B). Animals were healthy in all concentrations for the duration of the experiments. Gametes spawned in the highest concentration (10^{-5} *M*) were viable and oocytes were successfully fertilized, forming swimming trochophores within 20 h.

The spawning data for fluoxetine were pooled for 1996–1997. Fluoxetine was also an effective inducer of spawning in both sexes. A high, statistically significant percentage of males spawned from 5×10^{-7} to 5×10^{-4} *M* compared with negative controls (Fig. 3A, B). Females had a much narrower range of sensitivity, spawning at significant percentages only in 5×10^{-6} and 10^{-5} *M*. As in fluvoxamine, most males spawned within the first hour (Fig. 4A), but females lagged, achieving maximum spawning only after the second hour (Fig. 4B). Mussels exposed to high concentrations (10^{-3} *M*) of fluoxetine looked unhealthy, and sperm spawned in 10^{-3} to 5×10^{-5} *M* were not motile. But, sperm recovered motility when placed in fresh lake water. A single attempt to fertilize oocytes spawned in fluoxetine (10^{-5} *M*) was not successful.

In contrast to fluvoxamine and fluoxetine, paroxetine was only marginally effective at inducing spawning. Males spawned significantly in 10^{-6} and 10^{-5} *M*, but the highest percentage was only 50% in 10^{-6} *M* (Fig. 5A). Females did not show significant spawning at any concentration of paroxetine, although at 10^{-5} *M*, 2 of 8 females spawned. Of the 12 males that spawned in paroxetine, 6 spawned within the first hour, and 5 spawned within 2 hours. Females took at least 3 hours to spawn. Mussels exposed to high concentrations (10^{-4} *M*) of paroxetine released mucus from their siphons, and some of the sperm spawned at 10^{-5} *M* were not motile. No attempt at fertilizing paroxetine-stimulated spawned oocytes was made.

Discussion

Fluvoxamine, fluoxetine, and paroxetine increase 5-HT neurotransmission in vertebrates by inhibiting 5-HT reup-

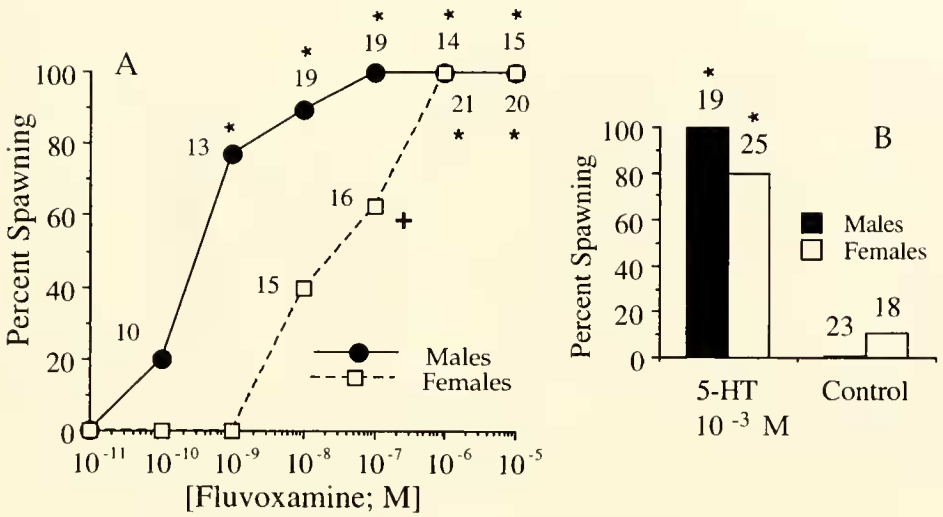

Figure 1. Dose-response experiments with fluvoxamine, June 1997. (A) Percent spawning of *Dreissena polymorpha* in different concentrations of fluvoxamine. Numbers of spawnable mussels tested are adjacent to symbols. *: $P < 0.0001$; +: $P < 0.003$ compared with negative controls. (B) Positive (5-HT) and negative (0.1% ETOH) controls for dose-response experiments. *: $P < 0.0001$. The sample sizes for females at 10^{-11}, 10^{-10}, and 10^{-9} *M* were 10, 10, and 7, respectively.

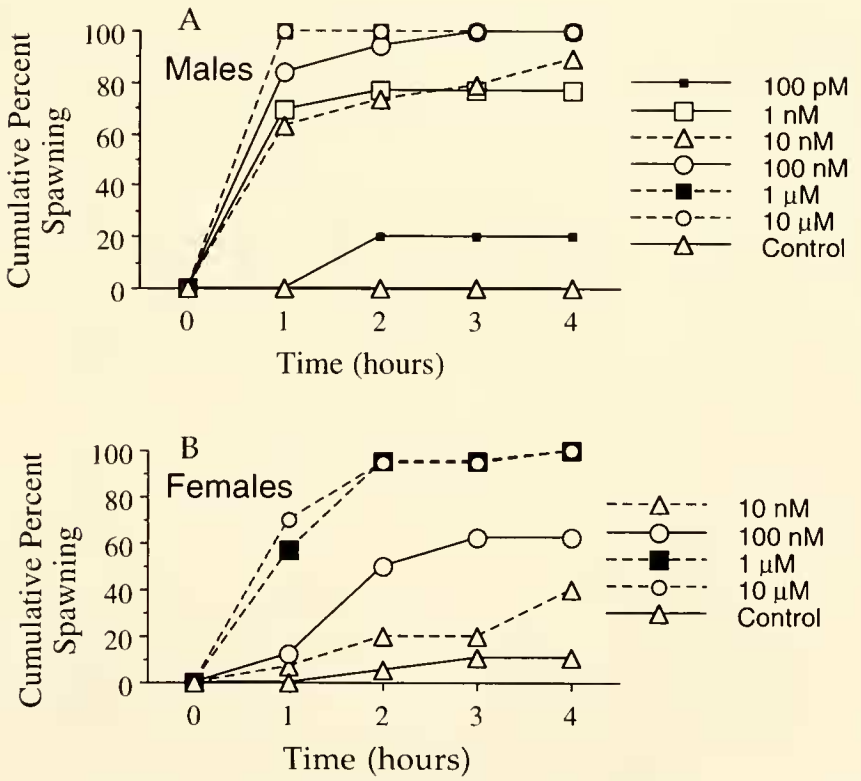

Figure 2. Cumulative percent spawning of zebra mussels over a 4-h period in different concentrations of fluvoxamine in (A) males and (B) females. Sample sizes (n) in each group range from 10 to 23 (for males) and from 15 to 21 (for females).

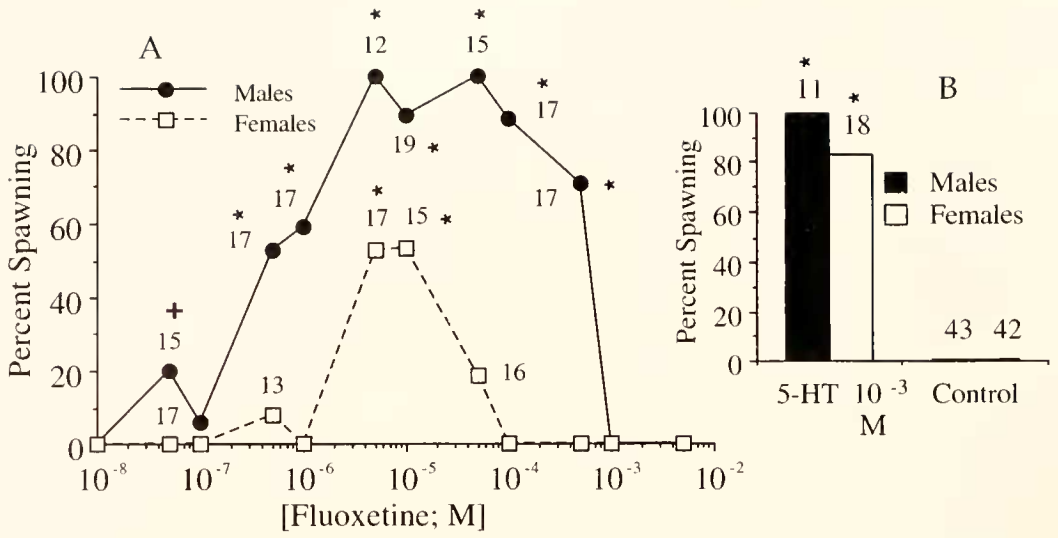

Figure 3. Dose-response experiments with fluoxetine. Data from July 1996 and June 1997 were pooled. (A) Percent spawning of *Dreissena polymorpha* in different concentrations of fluoxetine. Numbers of spawnable mussels tested are adjacent to symbols. *: $P < 0.0001$; +: $P < 0.05$ compared with negative controls. (B) Positive (5-HT) and negative (lake water) controls for dose-response experiments. *: $P < 0.0001$.

take transporters in synaptic clefts (Garcia-Colunga *et al.*, 1997). In molluscs, these compounds affect spawning (bivalves), induction and potentiation of parturition (fingernail clams; Fong *et al.*, 1998), and induction of metamorphosis (gastropod larvae; Couper and Leise, 1996). In other invertebrates such as crayfishes, ovarian growth is stimulated by both 5-HT and fluoxetine, and the latter potentiates the 5-HT effect (Kulkarni *et al.*, 1992). Similarly, fluoxetine potentiates 5-HT-stimulated testicular growth in male fiddler crabs (Sarojini *et al.*, 1993).

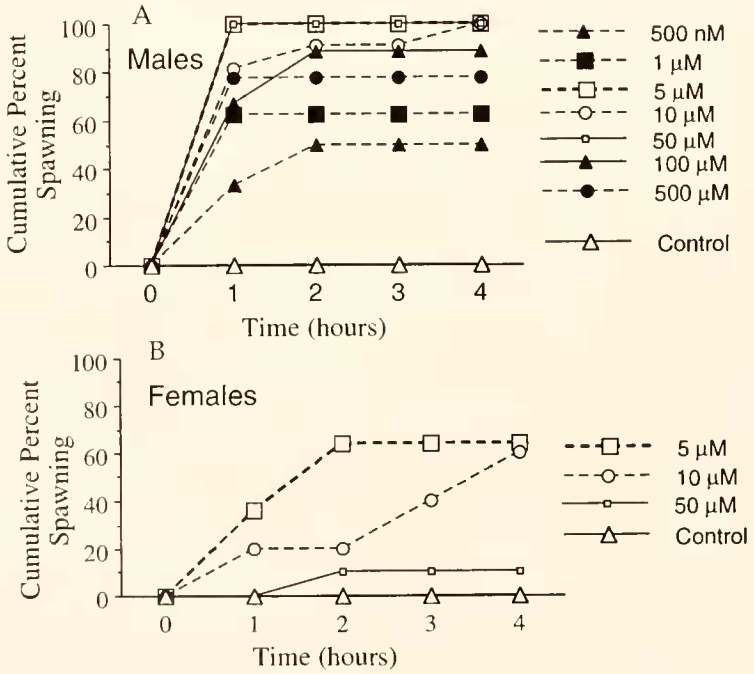

Figure 4. Cumulative percent spawning of zebra mussels over a 4-h period in different concentrations of fluoxetine in (A) males and (B) females. Sample sizes (n) in each group range from 7 to 17 (for males) and from 5 to 17 (for females). Data available for 1997 only.

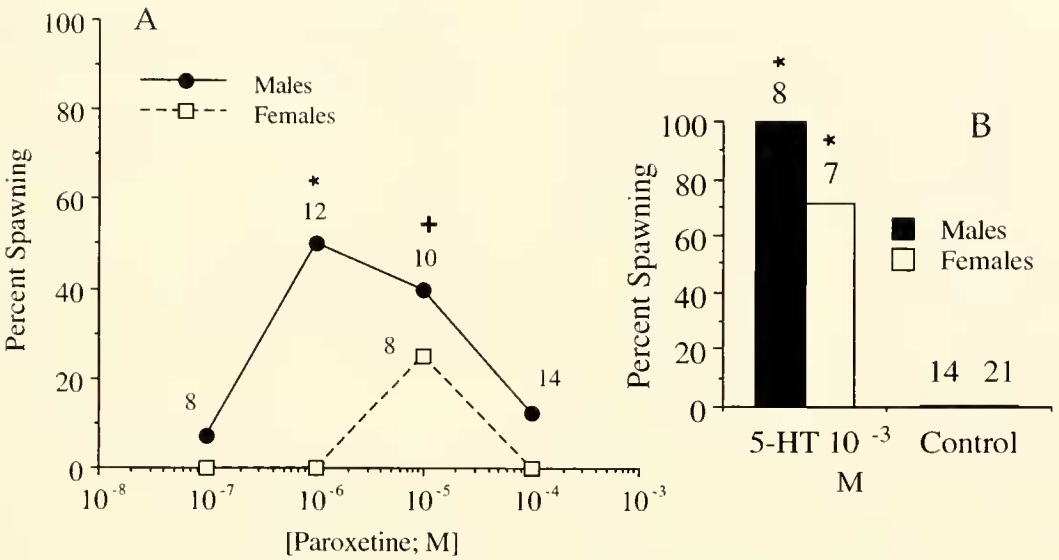

Figure 5. Dose-response experiments with paroxetine, June 1997. (A) Percent spawning of *Dreissena polymorpha* in different concentrations of paroxetine. Numbers of spawnable mussels tested are adjacent to symbols. *: $P < 0.004$; +: $P < 0.02$ compared with negative controls. (B) Positive (5-HT) and negative (lake water) controls for dose-response experiments. *: $P < 0.0002$.

As to the actual mechanism of the spawning induced in mussels by the three tested compounds in my study, two questions arise: (1) Are these compounds working as reuptake inhibitors or as ligands? and (2) How do these compounds gain entry into the animal? At this point, it is not known whether, in zebra mussels, fluoxetine, fluvoxamine, or paroxetine act as SSRIs or as ligands. Molluscan 5-HT reuptake mechanisms have been shown in snails (Osborne *et al.*, 1975) and in squids (Feldman and Dowdall, 1973). Furthermore, *Spisula* oocytes are known to have 5-HT receptors on their membranes, and 5-HT induces germinal vesicle breakdown (GVBD) (Hirai *et al.*, 1988). However, application of either fluvoxamine (10^{-5} *M*) or fluoxetine (10^{-6} and 10^{-5} *M*) to stripped *Spisula* oocytes did not induce GVBD, but 5-HT (10^{-5} *M*) did (unpubl. data). Thus there is no evidence that these compounds act as 5-HT receptor ligands in this well-known bivalve system. In lobsters, Huber *et al.* (1997a) showed reuptake of 5-HT in thoracic nerve roots and blockage of reuptake by fluoxetine. In this system, 5-HT stimulates subordinate animals to engage in fighting against dominants by reducing their willingness to retreat (Huber *et al.*, 1997b). However, injection of fluoxetine alone has no effect on fighting behavior; in fact, concommitant administration of 5-HT and fluoxetine decreased 5-HT-induced agonistic behavior (Huber *et al.*, 1997b). These authors suggested that long-term application of fluoxetine is necessary to mimic 5-HT-induced responses in their system, as is the case in the clinical treatment of depression in humans. In contrast, evidence that fluoxetine acts as a 5-HT receptor ligand has recently been reported (Ni and Miledi, 1997). In their study, fluoxetine bound to and inhibited 5-HT_{2C} receptors in *Xenopus* oocytes. There is additional evidence of fluoxetine's affinity for other neurotransmitter receptors. Garcia-Colunga *et al.* (1997) showed that fluoxetine blocks both muscle and neuronal nicotinic acetylcholine receptors in a voltage-dependent and noncompetitive fashion.

If or how the tested compounds gain entry into zebra mussels is also unknown. In fact, it is not known how externally applied 5-HT itself gains access to zebra mussels. Ram *et al.* (1993) suggested the possibilities that an external receptor is present or that 5-HT is internalized to act directly on the gonad. The concentrations of the fluvoxamine, fluoxetine, and paroxetine necessary to induce spawning in zebra mussels are many orders of magnitude lower than that of 5-HT itself (10^{-4} *M*), and are in the range of 5-HT concentrations (10^{-9} *M*) that induce inotropic (Gaddum and Paasonen, 1955) and inhibitory (Wilkens and Greenberg, 1973) effects on molluscan hearts.

To reiterate, whether fluvoxamine, fluoxetine, and paroxetine are acting as bona fide SSRIs or as 5-HT receptor ligands is unknown, as is their mode of entry. Radioligand binding studies would help elucidate if and where these drugs, when externally applied, are internalized by zebra mussels.

There is intense interest in SSRIs in mammalian vertebrates not only in the treatment of depression, but also in the treatment of conditions such as convulsive seizures (Pasini *et al.*, 1996), obsessive-compulsive behaviors associated with Tourette's syndrome (Eapen *et al.*, 1996),

and obesity in non-insulin-dependent diabetics (Daubresse *et al.*, 1996). However, the actions of putative SSRIs in invertebrates are poorly understood, even though 5-HT is a widely occurring biogenic monoamine that has been identified in a large number of invertebrates including cnidarians, platyhelminthes, nemerteans, annelids, arthropods, and especially molluscs (Welsh and Moorhead, 1960; Fujii and Takeda, 1988; Sandeman *et al.*, 1988; Linn and Roelofs, 1993).

Although some authors report enhanced fertilizability of bivalve oocytes in 5-HT (Juneja *et al.*, 1993), others report that high concentrations of 5-HT have toxic effects on zebra mussel gametes (J. Lynn, pers. comm.; pers. obs.). In the present experiments, toxic effects on mussels were noticed at high concentrations of fluoxetine and paroxetine. However, these effects were observed after most, if not all, animals in each group had already spawned. Thus the toxicity is not believed to have stimulated the spawning. The reduction in sperm motility observed in fluoxetine and paroxetine may be due to the direct action of the drugs on the sperm, since sperm transferred to fresh lake water usually recovered their motility. The mucus release seen in mussels exposed to paroxetine has also been observed in fingernail clams exposed to the same drug (pers. obs.).

Other than algal and gamete extracts (Ram *et al.*, 1996), fluvoxamine, fluoxetine, and paroxetine are the only compounds that stimulate spawning in zebra mussels which are not well-known 5-HT receptor ligands. Moreover, the lowest concentrations of any compound previously known to induce spawning in zebra mussels was 10^{-8} *M* metergoline in males (Fong *et al.*, 1994b) and 10^{-5} *M* ergotamine in females (Ram *et al.*, 1996). In the present study, fluvoxamine induced spawning in males at 10^{-10} *M* and in females at 10^{-8} *M*. Thus fluvoxamine is the most powerful spawning inducer identified not only for zebra mussels, but also for any bivalve yet tested. As powerful as fluvoxamine is, the sensitivity to this drug varies between species. The surf clam *Spisula solidissima*, one of the first bivalves to be induced to spawn with 5-HT, has a serotonin pharmacological profile similar to that of zebra mussels (Kadam *et al.*, 1991). Fluoxetine (10^{-5} and 10^{-4} *M*) induces some spawning in surf clams, but fluvoxamine (10^{-6} and 10^{-5} *M*) does not (unpubl. obs.). Although 5-HT is used to induce spawning in economically important bivalves, its high cost can prohibit its use in developing countries. Further testing of economically important bivalves with these and other putative SSRIs could uncover compounds that would induce spawning at much lower concentrations, and hence be more economically feasible, than 5-HT.

The use of fluvoxamine, fluoxetine, and paroxetine provides a possible method of stimulating serotonin-mediated responses in invertebrates without injection or external application of 5-HT or its ligands. Further experiments are needed to verify that these drugs act as SSRIs and to ascertain how widespread 5-HT reuptake transporters are in molluscs and other invertebrates.

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